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The Formation and Structural Investigation of Galacturonides from a Galactoglucomannan and a Galactomannan

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THE FORMATION AND STRUCTURAL INVESTIGATION OF GALACTURONIDES FROM A GALACTOGLUCOMANNAN AND A GALACTOMANNAN

A thesis submitted by

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SUMMARY

The present investigation is the pioneering work on the formation of a galacturono-galactoglucomannan from the neutral polymer which was isolated from the chlorite liquor of an Engelmann spruce holocellulose; the neutral hemicellulose contained D-galactose, D-glucose, and D-mannose residues in a molar ratio of 1.2:0.8:3. Some of the D-galactose groups in the galactoglucomannan preparation were oxidized to D-galacturonic acid units in two successive stages. The C6 positions were converted to aldehyde groups by oxidation with a commercial galactose oxidase and subsequently to carboxyl groups by oxidation with an alkaline iodine solution. Three two-stage oxidations were required to oxidize approximately 50% of the D-galactose residues to D-galacturonic acid units.

This investigation also marks the first time a galacto-urono-galactomannan has been prepared from a neutral galactomannan polysaccharide. Employing one two-stage oxidation, approximately 40% of the D-galactose residues were converted to D-galacturonic acid units.

The oxidized polymers were subjected to partial acid hydrolyses, and the uronides were isolated. One acidic component was isolated from the hydrolysate of oxidized guaran, and six acidic components were obtained from the hydrolysate of oxidized galactoglucomannan. The component from oxidized guaran and the major component from oxidized galactoglucomannan were the same aldobiouronic acid, 6-O-α-D-galactopyranosyluronic acid-D-mannopyranose (A1). Other acidic compounds from oxidized galactoglucomannan were: O-α-D-galactopyranosyluronic acid-D-glucopyranose (A2), O-α-D-galactopyranosyluronic acid-O-D-glucopyranosyl-D-mannopyranose (A4), and O-D-galactopyranosyluronic acid-D-galactopyranose which was believed to have come from a galactan impurity. Of the two remaining acidic components from the hydrolysate of oxidized galactoglucomannan, one appeared to be a mixture of aldotriouronic acids the exact nature of which could not be demonstrated

because of insufficient sample; and the yield of the other was too small to conduct meaningful experimental tests.

Three new compounds were isolated and partially characterized in this investigation; they are A1, A2, and A4. Acids A2 and A4 are especially noteworthy since they represent the first direct confirmation of a galactose-glucose linkage in a galactoglucomannan polymer. Acid A4 is also the most thoroughly characterized trihetero-trisaccharide thus far obtained from galactoglucomannans, and it strongly supports the hypothesis of a true triheteropolymeric galactoglucomannan.

INTRODUCTION

When acid chlorite holocelluloses are prepared from the wood of gymnosperms, a mixture of hemicelluloses is dissolved in the chlorite liquor. This extract can be fractionated by repeated precipitation with barium hydroxide or Fehling solution; and the purified, precipitated material contains galactose, glucose, and mannose residues in a ratio of approximately 1:1:3 (1, 2). A similar material can be obtained by extracting acid chlorite holocelluloses from gymnosperms with aqueous potassium hydroxide. The same purification procedure is employed, and the approximate ratio of galactose, glucose, and mannose is 1:1:3 (3, 4). This galactose-rich, water-soluble fraction is called galactoglucomannan hemicellulose. After the galactoglucomannan is removed, extraction of the holocellulose residue with sodium hydroxide removes a galactose-poor, water-insoluble fraction. The purified barium hydroxide precipitate of this fraction contains galactose, glucose, and mannose units in a ratio of approximately 0.1-0.2:1:3 (4) and is commonly called glucomannan hemicellulose.

There are two schools of thought concerning the existence of a galactoglucomannan hemicellulose. One group of investigators has presented evidence which indicates that a triheteropolymer really exists, while other workers in this field indicate that the material called galactoglucomannan is actually a mixture of polysaccharides. The evidence for these points of view will be reviewed in the following section.

HISTORICAL REVIEW

In their initial investigation of softwood galactoglucomannans, Hamilton, Partlow, and Thompson (5) employed kraft pulps and a western hemlock holocellulose; and the isolated material could not be resolved further by any of

five different fractionation procedures. Therefore, Hamilton and coworkers (6) proposed the existence of a true triheteropolymer galactoglucomannan. These investigators also methylated the polysaccharide and, upon hydrolysis, obtained the products enumerated in Table I. On the basis of their methylation and periodate-oxidation data, Hamilton and coworkers set forth a tentative structure for this new hemicellulose; the structure is illustrated schematically in Fig. 1.

TABLE I

HYDROLYSIS PRODUCTS OF A METHYLATED POLYSACCHARIDE (6)

Compound	Relative Mole, %
2,3-di-O-methyl-D-mannose	10.0
2,3-di-O-methyl-D-glucose	3.2
2,3,6-tri-O-methyl-D-mannose	55.0
2,3,4,6-tetra-O-methyl-D-galactose	15.3
2,3,6-tri-O-methyl-D-glucose	13.2
2,3,4,6-tetra-O-methyl-D-mannose	3.2

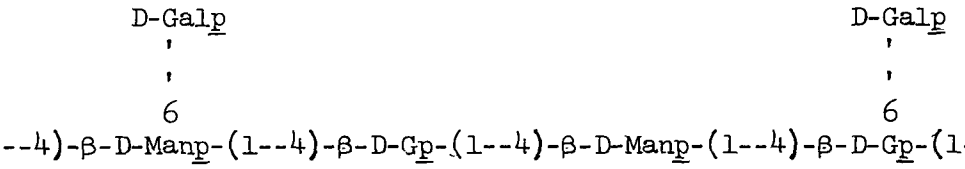

Figure 1. Proposed Structure for Galactoglucomannan (4).

In subsequent investigations, Timell, et al., (7, 8, 9) completely corroborated the structural work carried out by Hamilton and coworkers. Large amounts of 2,3,6-tri-O-methyl-D-glucose and 2,3,6-tri-O-methyl-D-mannose were isolated from the acid hydrolysates of the methylated polysaccharides indicating that they were composed of 1,4-linked glucopyranose and mannopyranose units. The total number of 2,3-di-O-methyl-D-glucose and 2,3-di-O-methyl-D-mannose monomers was approximately equal to the number of 2,3,4,6-tetra-O-methyl-D-galactose units; this indicates that galactose residues terminate branches bonded by a 1,6-linkage.

The periodate oxidation data confirmed the results of methylation; 1.20 moles of periodate were consumed per hexose unit as expected from the proposed structure in Fig. 1. After partial acid hydrolysis, a number of oligosaccharides were isolated and identified; and the linkages indicated that the backbone residues of the galactoglucomannan polymer are joined by β-1,4-linkages.

The structural studies of galactoglucomannan polymers indicated that side chains are terminated by D-galactose residues and are linked to mannose units of the backbone by a 1,6-linkage. The investigation of Schwarz and Timell (9) elucidated the nature of the galactose-mannose bond. They isolated and identified two oligosaccharides composed of galactose and mannose residues, and each one contained a single galactose unit which was attached to mannose by an α-1,6-linkage. Timell indicated that two structural arrangements of the branch are possible, and these are shown in Fig. 2. He pointed out that, on methylation and hydrolysis, alternative B would yield 2,3,4-tri-O-methyl-D-mannose; and since this monomer has not been observed, arrangement A must apply.

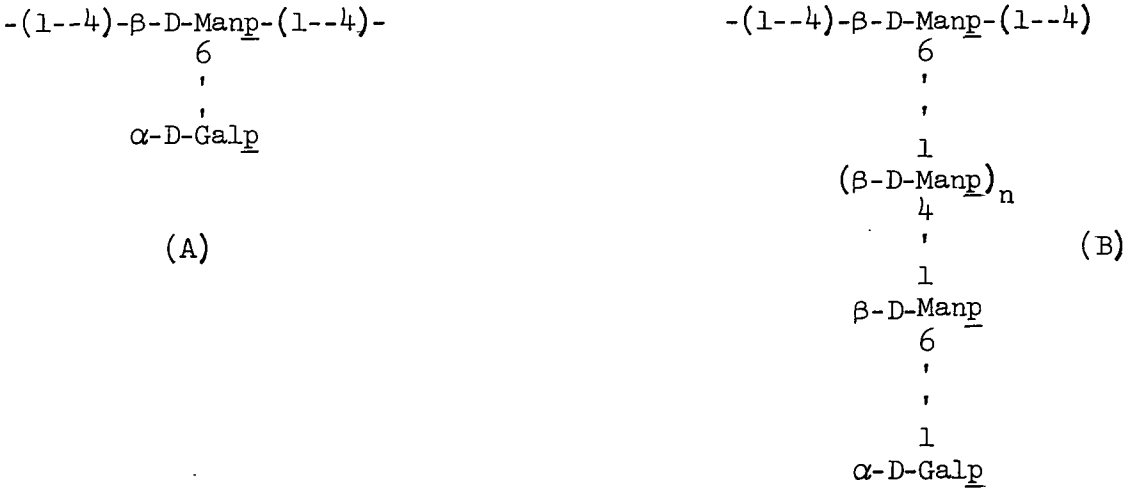

Figure 2. Possible Mannose-Linked Branch Structures in Galactoglucomannan (4)

The nature of the branches attached to glucose in a galactoglucomannan polymer is not well understood, and structural investigations may be interpreted to indicate that side chains are attached to glucose at C6 and are terminated by galactose residues. Since galactoglucomannan polymers contain larger amounts of galactose than glucomannan polymers and display less negative specific rotation, the galactose linkages are probably of the α-configuration. Three conceivable side chains are illustrated in Fig. 3; and although the sugar residues positioned between the galactose unit and the polymer backbone are glucose units, mannose units are also possible. Upon methylation and hydrolysis, Alternative C would yield a monomer methylated in the 2,3, and 4 positions; and since a compound of this type has not been observed, the existence of Structure C is doubtful. Side-chain structures similar to Alternatives A and B appear more feasible.

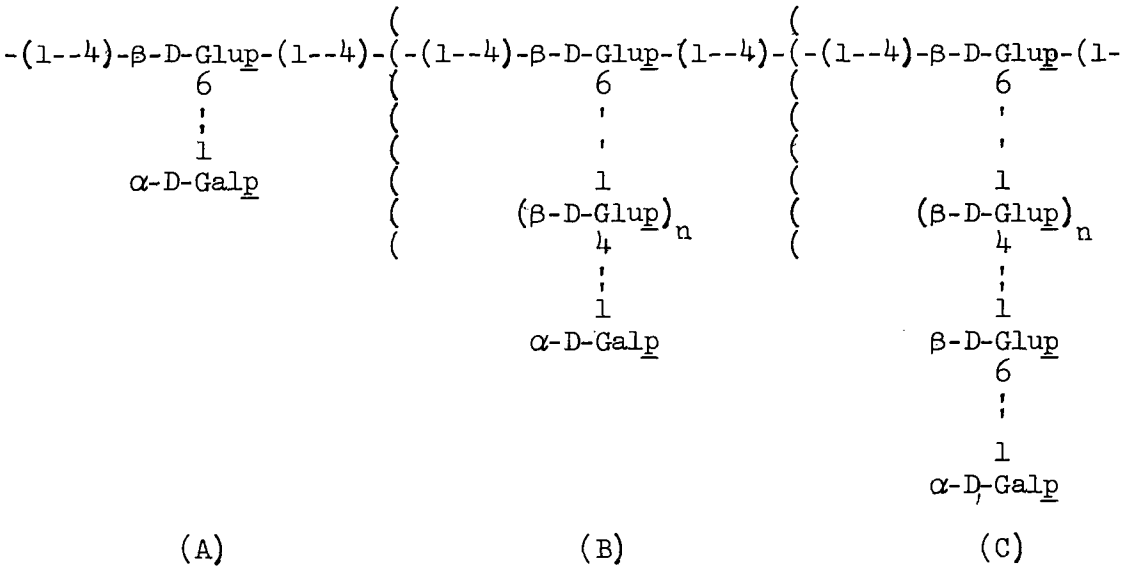

Figure 3. Possible Glucose-Linked Branch Structures in Galactoglucomannan

Timell (4) suggests that because softwood galactoglucomannans are water soluble, have constancy of composition upon repeated fractionation, and are apparently homogeneous on free boundary electrophoresis and ultracentrifugation, the existence of a true heteropolymer is strongly indicated. Mills and Timell (8), working

with galactoglucomannans, have recently isolated a chromatographically homogeneous trimer containing galactose, glucose, and mannose. Although this compound was not characterized, it constitutes evidence for the triheteropolymeric nature of water-soluble galactoglucomannans.

Publications by Meier and coworkers indicate that they doubt the existence of galactoglucomannans. Croon, Lindberg, and Meier (10) demonstrated that glucomannan preparations often contain galactose and xylose residues which could not be removed by fractionation; they also showed that small amounts of lignin were present in the glucomannan preparations. Since all of the xylose and most of the galactose residues could be removed by complete delignification, these investigators indicated that different polysaccharides such as glucomannans, xylans, and galactans might be linked chemically to lignin.

Meier (1) studied the hemicellulose fraction which was soluble in the chlorite liquor of a Norway spruce holocellulose and subjected a galactoglucomannan to a partial acid hydrolysis. He isolated a dimer, 6-O-α-D-galactopyranosyl-D-mannopyranose, and a trimer, 6-O-α-D-galactopyranosyl-4-O-β-D-mannopyranosyl-D-mannopyranose, in this study. The identification of these compounds could be interpreted as evidence for the existence of a galactoglucomannan polymer; however, Meier implied that the presence of a galactomannan in addition to the previously known glucomannan is more likely.

WORKING HYPOTHESIS

When all the data of the previous section are considered, there is strong inference for the existence of a triheteropolymer galactoglucomannan; however, its structure has not been definitely established. Proof of the existence of a true triheteropolymer galactoglucomannan will be obtained when a fragment of the

polymer containing galactose, glucose, and mannose is isolated and characterized. Therefore, the hypothesis for this study is given in the following statement: a true triheteropolymer galactoglucomannan exists in the wood of gymnosperms with D-galactose groups attached directly to D-glucose residues. The fact that D-galactose groups are linked to D-mannose units in the polymer has been established and is therefore not included in the statement of the hypothesis.

ANALYSIS OF THE PROBLEM

Proposed structures are often verified by isolating and identifying the oligosaccharides found after acid hydrolysis of the polysaccharide in question. However, the galactose linkages in the galactoglucomannan polymer are quite labile to acid hydrolysis (4, 6), and oligosaccharides containing galactose are difficult to obtain. Although the more abundant galactose-mannose linkage has been found, no dimer containing galactose and glucose has ever been isolated and characterized.

When the C6 position of a glycoside is oxidized from a primary alcohol group to a carboxyl group, the stability of the glycosidic bond to acid hydrolysis is increased (11, 12). Hypotheses based on steric effects (13) and inductive effects (14-16) have been advanced to explain the strong resistance of aldobiouronic acids to acid hydrolysis. Inductive effects have also been used to explain why aldotriouronic acids are more stable to acid hydrolysis than the analogous neutral trimers.

The enzyme galactose oxidase was discovered by Cooper, et al. (17); and in a more recent study Amaral, et al. (18) showed that it is a copper enzyme containing one mole of copper per mole with a molecular weight of approximately 75,000. Avigad, et al. (19) investigated the action of this enzyme on D-galactose and numerous D-galactosides. They found that the enzyme specifically oxidizes the

C6 position of the galactose units to the aldehyde in all the compounds tested, albeit at different rates, according to the following sequence:

$$\text{D-galactose} + O_2 \rightarrow \text{D-galacto-hexodialdose} + H_2O_2 \quad (20).$$

The hydrogen peroxide formed by the action of the enzyme significantly inhibits the efficiency of the oxidase when present in concentrations greater than 3×10^{-3} molar (19). However, the hydrogen peroxide can be removed from the reaction system by the use of an enzyme called catalase which decomposes it to water and gaseous oxygen (21).

It appeared feasible that the C6 positions of the D-galactose units in galactoglucomannans could be oxidized to aldehyde groups by galactose oxidase and subsequently to carboxyl groups by employing a suitable oxidizing agent. If a selective oxidation of the C6 positions of D-galactose were accomplished, a new galacturono-galactoglucomannan would be formed; and a graded acid hydrolysis would yield a spectrum of acidic compounds. It is quite probable that characterization of these uronides would elucidate the structure of the galactoglucomannan polysaccharide.

One of the acidic dimers expected from the partial acid hydrolysis of galacturono-galactoglucomannan is 6-O-α-D-galactopyranosyluronic acid-D-mannopyranose. Since guar gum is a straight-chain mannan with single member D-galactose branches which are joined by α-1,6 linkages (22, 23), an authentic sample of this aldobiouronic acid could be obtained by oxidation of the C6 positions of the D-galactose residues to carboxyl groups and subsequent hydrolysis.

Therefore, the objectives of this study were: a) the formation of galacturonides from galactoglucomannan and from guaran, and b) the isolation and characterization of the acidic dimers and/or trimers obtained from the partial acid hydrolysis of the oxidized polymers.

EXPERIMENTAL RESULTS

A 44-pound, debarked log of Engelmann spruce was obtained from Colorado through the United States Forest Service. The log was split into four pieces and chipped in an automatic chipper; subsequently about 30 pounds of unconditioned chips were made into wood meal using a Wiley mill with Screen 1. The meal was screened in a large shaker to remove the very coarse and very fine fractions, and the wood which passed through a 4-mesh screen but not through a 20-mesh screen was saved for the holocellulose preparation. Wood meal rather than wood chips was used to hasten the delignification of the holocellulose, and the screening was done to increase the uniformity of delignification.

HOLOCELLULOSE PREPARATION

In order to keep polysaccharide degradation at a minimum, a room-temperature, acid-chlorite holocellulose was prepared in a manner similar to that described by Thompson and Kaustinen (24, 25). The airdry wood meal, 9600 g. ovendry (o.d.), was chlorited in four separate batches using 32-liter battery jars as reaction vessels. In order to gain better penetration of the chlorite liquor into the wood, measured amounts of meal and liquor were placed in vacuum desiccators; and vacuum was applied for 25 minutes. The contents of the desiccators were placed in the reaction vessels, and the acid was added to initiate the formation of chlorine dioxide. It was observed that the reaction was exothermic and that the reaction mixture reached a temperature of approximately 60°C. before cooling. Each batch of holocellulose was treated in the same manner, and the conditions are given in Table II.

Four days after the holocellulose preparation was started, an additional 1275 g. of sodium chlorite were dissolved in 2 liters of deionized water and added

to each vessel. A second, similar supplement was made to the reaction vessels four days later, and the terminal chlorite-to-wood (o.d.) ratio was 2.1.

TABLE II

CONDITIONS OF HOLOCELLULOSE PREPARATION

Initial chlorite concentration, g./liter	100
Glacial acetic acid per vessel, ml.	35
Liquor-to-wood (o.d.) ratio (V:W)	10.6
Wood meal (o.d.) per vessel, g.	2400
Initial chlorite-to-wood (o.d.) ratio (W:W)	1.06

The holocellulose reaction mixture was quenched after 14 days by filtering the holocellulose on a large sintered-glass funnel and placing the delignified wood meal in deionized water overnight. The chlorite liquor was placed in large glass jars and retained for further study. After the holocellulose was steeped in water overnight, washed twice with deionized water, and placed in methanol overnight, it was filtered on large sintered-glass funnels and spread out to dry. The properties of the holocellulose are illustrated in Table III.

TABLE III

PROPERTIES OF HOLOCELLULOSE[a]

Moisture content, %	9.8
Klason lignin, % (26)	0.7
Ash, % (27)	3.0

[a]The holocellulose was extracted with 24% potassium hydroxide (see Appendix I).

ISOLATION AND PURIFICATION OF GALACTOGLUCOMANNAN

Since the galactoglucomannan used in this study was to be obtained from the 100 liters of chlorite liquor, it was placed in dialyzing tubing and dialyzed against deionized water to remove the sodium chloride. After two weeks, addition of silver nitrate solution to a sample of liquor resulted in a very light precipitation of silver chloride which indicated that most of the chloride had been removed. The dialyzed chlorite liquor, about 130 liters, was concentrated to a volume of about 3 liters using a large cyclone evaporator.

The concentrated chlorite liquor contained a small amount of insoluble material which was removed by centrifugation, and the supernatant was diluted with 2 volumes of saturated barium hydroxide solution. The precipitation of the barium complex was complete after this addition. The precipitate was collected by centrifugation, made into a paste with deionized water, and acidified to pH 6 with glacial acetic acid whereupon most of the precipitate dissolved; and the insoluble material was separated by centrifugation. After dialyzing the supernatant against deionized water for a week, a dilute solution of sulfuric acid was added to a sample of the dialyzate; and a very light precipitate of barium sulfate formed which indicated that most of the barium was removed. The dialyzate was concentrated from 10 liters to 5 liters using a cyclone evaporator.

In order to purify the polymer preparation further, the concentrated dialyzate was diluted with an equal volume of freshly prepared Fehling solution. A very heavy precipitate immediately formed which was collected by centrifugation, made into a paste with deionized water, and acidified to pH 6 with glacial acetic acid. A very small amount of insoluble material remained and was centrifuged from the supernatant.

In an attempt to lessen the salt concentration, 4 volumes of absolute alcohol were added to the supernatant. A bluish-white, flocculent precipitate formed which was collected by centrifugation and dissolved in water. A very small amount of insoluble material was removed by centrifuging, and the supernatant was dialyzed to remove the ions from the polymer. However, it was found that not all of the blue color, characteristic of cupric ion, could be removed in this manner; therefore, the solution was thoroughly deionized with Amberlite IR-120 (H^+)* and freeze dried. Figure 4 illustrates the four-month isolation and purification of the galactoglucomannan.

CHARACTERIZATION OF GALACTOGLUCOMANNAN

The ovendry yield of galactoglucomannan was 14.5 g.; this was 0.15% of the ovendry wood meal. The polymer was a light yellow color, and had a specific rotation, $[\alpha]_D^{26}$ (water) of +2.8 degrees.

ULTRACENTRIFUGE

In order to check on the homogeneity of the galactoglucomannan preparation, a 1% aqueous solution of the polymer was examined by ultracentrifugation using a Spinco Model E analytical ultracentrifuge with a Schlieren optical system. Upon ultracentrifuging for 1-1/4 hours at 60,000 r.p.m., the galactoglucomannan gave a symmetrical bell-shaped pattern; this is a necessary but not sufficient condition to prove homogeneity.

FREE BOUNDARY ELECTROPHORESIS

In another test of homogeneity, the galactoglucomannan was subjected to free boundary electrophoresis using a Spinco Model H electrophoresis apparatus. A 2%

*Amberlite ion-exchange resins are manufactured by Rohm and Haas, Philadelphia.

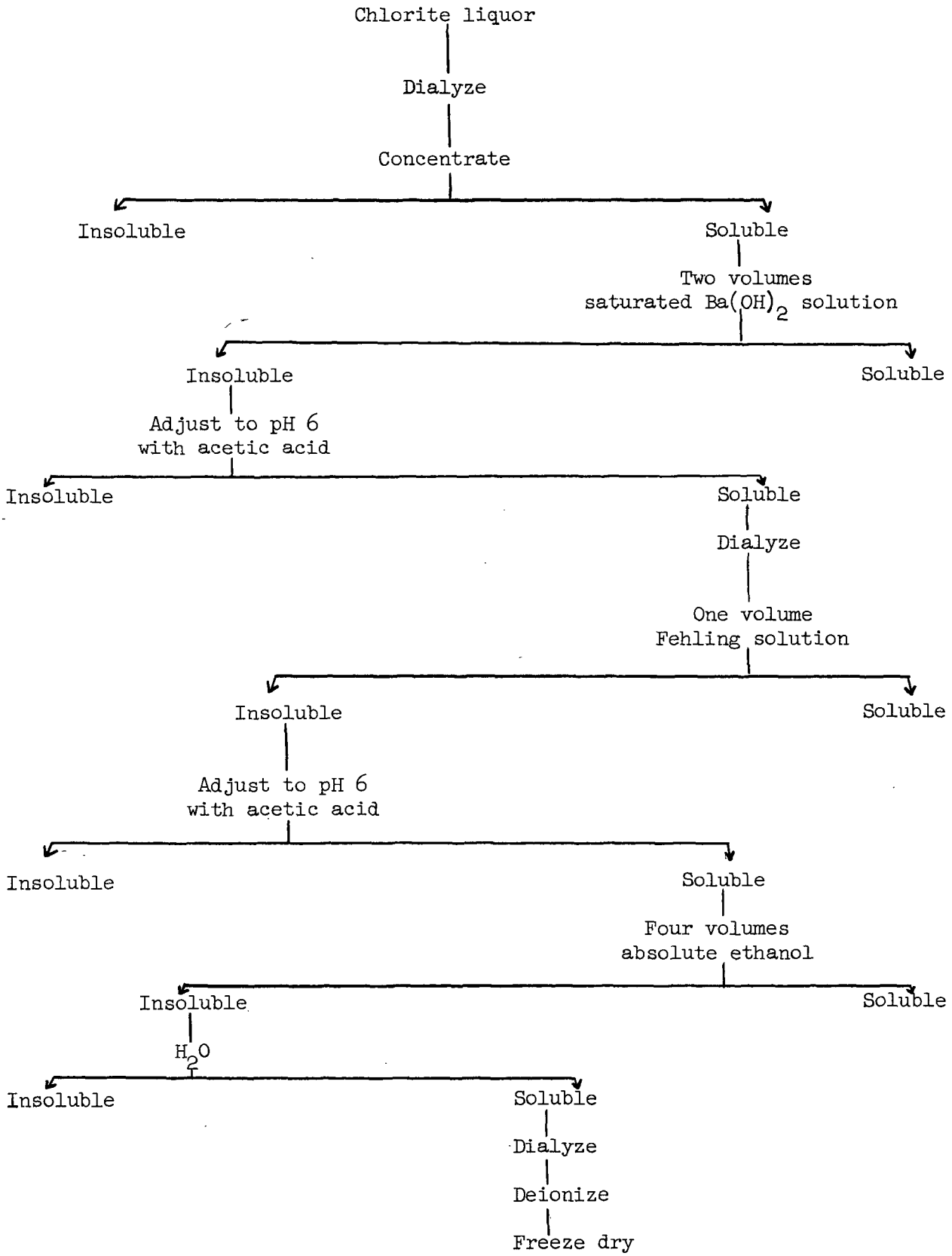

Figure 4. Isolation and Purification of Galactoglucomannan

solution of the polymer in borate buffer, pH 9.2, was treated with 13 milliamperes for 3-1/2 hours at 4.5°C. A sample was taken from the ascending side of the electrophoresis cell and another from the descending side. After deionizing with Amberlite MB-3(H^+, OH^-) and evaporating to dryness, the ascending sample was a light brown powder; and the descending sample was a white powder. Approximately 4 mg. of each sample were dissolved in 2 ml. of 4.5% sulfuric acid and heated in a household pressure cooker for one hour at 120°C. The hydrolysates were neutralized with barium carbonate, filtered on a coarse sintered-glass funnel with a cellulose pad, deionized with Amberlite IR-120 (H^+), and evaporated to dryness. About 25 μg. of each sample were spotted on a sheet of Whatman No. 1 chromatography paper with knowns and developed for 24 hours with Solvent C followed by elution with Solvent B for another 12 hours (see Appendix III for chromatography data). The migrating compounds were detected with silver nitrate dip, and the ascending sample showed evidence of trace amounts of glucuronic and galacturonic acids while the descending did not.

Quantitative sugar analyses were performed on each sample using the method described by Saeman, et al., (28). The ascending sample had a molar ratio of xylose, arabinose, galactose, glucose, and mannose of 0.2:0.2:1.7:0.7:3 while the descending sample contained no detectable xylose or arabinose and had a molar ratio of galactose, glucose, and mannose of 0.9:0.7:3. Assuming the descending sample contained pure galactoglucomannan, the large amount of galactose found in the ascending sample indicated that the polymer preparation contained a 6.5% galactan contaminant as well as small amounts of xylose and arabinose.

LIGNIN CONTENT

It has been demonstrated that glucomannan preparations often contain small amounts of lignin (10, 29). Therefore, the galactoglucomannan was analyzed for

lignin using an alkaline nitrobenzene oxidation (30) which is a very attractive procedure when only small samples or low lignin contents are available. The average analyses from duplicate determinations were: 0.40% vanillin and 0.028% syringaldehyde from which was calculated 1.80% lignin (30).

ACETONE-SOLUBLE MATERIAL

A sample of isolated galactoglucomannan, 56.5 mg. o.d., was extracted with 25 ml. of acetone overnight. The insoluble polymer was filtered from the supernatant, and the amount of residue was determined by evaporating to dryness. For control purposes, an equal volume of acetone was also evaporated and the residue determined. Approximately 0.6% of the galactoglucomannan was soluble in acetone; however, the soluble material was not investigated further.

MOLECULAR WEIGHT

The molecular weight of the galactoglucomannan was determined by osmometry of its acetate which was prepared by a modification of the method described by Carson and Maclay (31). The degree of substitution, 3, was determined according to the technique reported by Genung and Mallett (32). The acetylated polymer was dissolved in nitroethane and tested at four different concentrations in a Mechrolab High Speed Membrane Osmometer using Schleicher and Schuell 08 membranes. The experimentally determined degree of polymerization was 64 which corresponds to a molecular weight for the acetylated polymer of 18,400.

SUGAR CONTENT

The quantitative sugar analyses of the galactoglucomannan were conducted by the method of Saeman, et al. (28); and the molar ratio of galactose, glucose, and mannose was 1.2:0.8:3. Institute Method 25 was used to determine the uronic acid

in the polymer, and Institute Method 4 was used to analyze for ash. All analyses were carried out in duplicate and the results are shown in Table IV.

TABLE IV

COMPOSITION OF GALACTOGLUCOMANNAN

	Galactoglucomannan
Glucan, %	11.7
Mannan, %	44.3
Galactan, %	17.9
Xylan, %	0.9
Araban, %	1.3
Uronic acid, %[a]	8.6
Ash, %	0.8
Total, %	85.5

[a]Carbon dioxide evolution calculated as uronic acid.

PRELIMINARY OXIDATION EXPERIMENTS

IODOMETRIC OXIDATION

An iodometric method for measuring the reducing power of aqueous solutions was described by Macleod and Robison (33). The procedure was tried by oxidizing four duplicate samples of a galactose solution (2.5 mg. per ml.) for various times in a constant-temperature bath at 30 ± 1°C. Each reaction flask contained 1 ml. of galactose solution, 6 ml. of 0.02N iodine solution, and 0.4 ml. of 5% sodium carbonate solution; and duplicate blanks were determined for each reaction time to correct for the reducing power of the reagents exclusive of galactose. After the desired reaction time, each flask was treated with 1 ml. of 0.5N sulfuric acid;

and using starch indicator, the excess iodine was titrated with 0.005N sodium thiosulfate. A simple calculation showed the percentage of galactose oxidized, and these data were plotted against the times of reaction. It was found that after five minutes the iodine consumption leveled off which indicated that all the C1 positions in galactose were oxidized to carboxyl groups (33).

ENZYME OXIDATION OF GALACTOSE

Commercial galactose oxidase was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey; and its effect on galactose was investigated using small-scale experiments, as described in Appendix II. Duplicate samples containing galactose were oxidized for various times in a constant-temperature bath at 30 ± 1°C., and control samples were also run in duplicate under the same conditions. After the desired reaction time, the reducing power of the solutions was determined by the iodometric technique described previously. Some control samples were used to determine the reducing power of the reagents exclusive of galactose, while others were used to show that all the C1 positions of galactose were oxidized. The percent of C6 positions oxidized by the enzyme was calculated and plotted against reaction time. The results are shown in Fig. 5.

ENZYME OXIDATION OF GLUCOSE AND MANNOSE

Glucose and mannose solutions were prepared; and duplicate samples were subjected to small-scale enzymatic oxidations, as described in Appendix II. Control samples were also run in duplicate; and after the desired reaction time, the reducing power of the various samples was determined by the iodometric technique. It was found that the galactose oxidase had no effect on the C6 positions of either sugar.

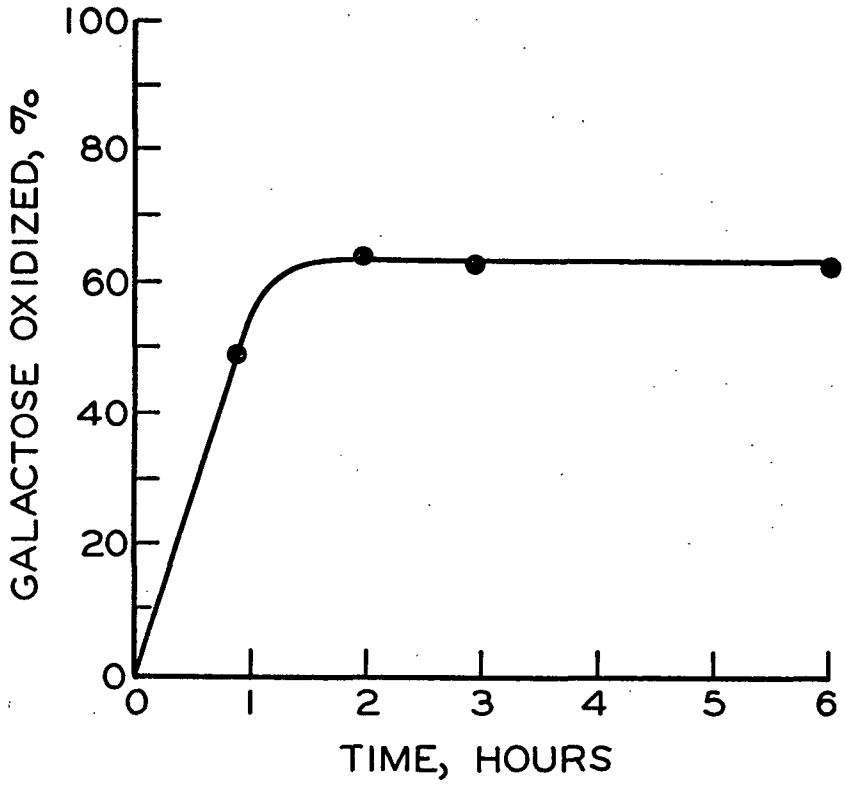

Figure 5. Oxidation of Galactose by Galactose Oxidase

ENZYME OXIDATION OF GALACTOGLUCOMANNAN

The galactoglucomannan was oxidized with galactose oxidase in small-scale experiments, as described in Appendix II. Duplicate samples containing polymer as well as control samples with and without polymer were run for various times in a constant-temperature bath at 30 $\pm$ 1°C. When the desired reaction time had elapsed, the reducing power of the samples was determined by the iodometric method. If the reducing power of the reagents and the unoxidized polymer is substracted from the reducing power of the reaction mixture, the effect of the enzyme on the galactoglucomannan can be evaluated. Assuming the enzyme has no effect on the lignin debris, Fig. 6 illustrates the percent of galactose groups oxidized versus the time of the reaction.

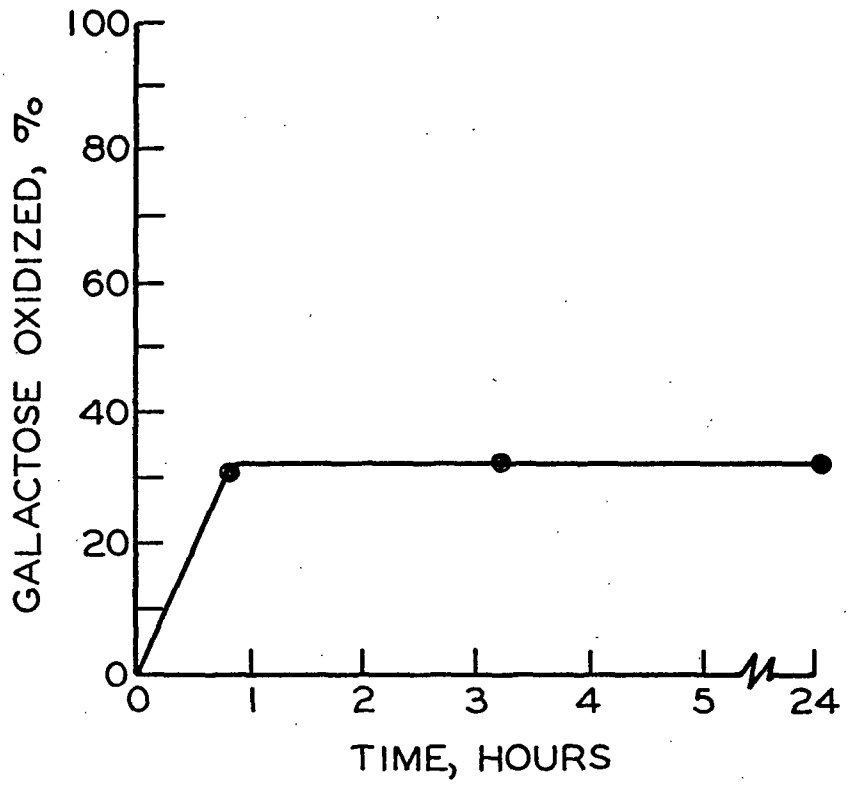

Figure 6. Oxidation of Galactoglucomannan by Galactose Oxidase

OXIDATION OF GALACTOGLUCOMANNAN

The oxidation of the galactoglucomannan, 5.0 g. o.d., was carried out in two stages with no isolation of polymer between stages. Initially, the galactoglucomannan was treated with an aqueous solution of galactose oxidase which oxidized some of the C6 positions of galactose to carbonyl groups. Subsequently, the reaction mixture was treated with an aqueous iodine solution which oxidized the carbonyl groups to carboxyl groups. The experimental conditions are illustrated in Table V.

TABLE V

EXPERIMENTAL OXIDATION CONDITIONS

STAGE 1:	
Temperature, °C.	30 ± 1
Time of enzymatic oxidation, hours	4
Concentration of galactose residue, mg./ml.	0.5
Concentration of galactose oxidase, units/mg. galactose	2.2
Concentration of catalase, units/mg. galactose	1200
Concentration of pH 7 buffer, ml./mg. galactose	0.6
STAGE 2:	
Temperature, °C.	30 ± 1
Time of iodometric oxidation, hours	4
Concentration of galactose residues, mg./ml.	0.2
Concentration of iodine, mg./mg. galactose	10
Concentration of sodium carbonate, mg./mg. galactose	13

After the second stage of the oxidation, the reaction mixture was heated in a boiling water bath for 15 minutes; and the reaction solution reached a maximum temperature of 85°C. This heating of the reagents served to vaporize much of the excess iodine and to insure the denaturization of the galactose oxidase. The reaction solution was cooled to room temperature; and after thorough deionization with Amberlite MB-3 (H^+, OH^-), the oxidized polymer was concentrated in a cyclone evaporator and isolated by freeze drying. Under these conditions, the recovery of oxidized polymer was approximately 96%.

A multiple oxidation technique was employed to reach as high a level of oxidation as was practicable. The once-oxidized polymer was subjected to second

and third two-stage oxidations under the conditions outlined above. After each of the three oxidations, about 100 mg. were sent to the Analytical Section for quantitative sugar (28) and uronic acid (Institute Method 25) analyses. The three samples of oxidized polymer were designated GGM 01, GGM 02, and GGM 03. The results of the analyses based on 100% carbohydrate (sugars and uronic acid) are shown in Table VI; the analyses of the unoxidized polymer, GGM, are also shown.

TABLE VI

ANALYSES OF OXIDIZED AND UNOXIDIZED POLYMER

	GGM	GGM 01	GGM 02	GGM 03
Glucan, %	13.8	12.2	12.6	12.9
Mannan, %	52.3	54.3	52.7	55.5
Galactan, %	21.1	14.1	10.8	9.8
Xylan, %	1.1	1.2	1.1	1.2
Araban, %	1.5	1.7	1.2	1.3
Uronic acid, %[a]	10.2	16.2	21.5	19.3

[a]Carbon dioxide evolution calculated as uronic acid.

The amount of galactose converted to uronic acid is not easily perceived from the data in Table V. Assuming that all the galactose lost is completely converted to uronide and that the initial 10.2% uronic acid is not lost, Table VII shows the theoretical and experimental percentages of uronic acid for each sample. All the values in the table were calculated on the basis of 100% carbohydrate (sugars and uronic acid).

TABLE VII

COMPARISON OF EXPERIMENTAL AND THEORETICAL URONIC ACID[a]

	Galactan, %	Uronic Acid, %	
		Theoretical +10.2	Experimental
GGM	21.1	10.2	10.2
GGM 01	14.3	16.9	16.2
GGM 02	10.8	20.3	21.5
GGM 03	9.8	21.3	19.3

[a]Carbon dioxide evolution calculated as uronic acid.

OXIDATION OF GUARAN

A sample of commercial guar gum was obtained from the Stein Hall Company, and the polymer was purified by Dugal and Swanson (34) as part of some recent work done at The Institute of Paper Chemistry. Approximately 10 g. of this polymer were purified by dissolving in 0.1N sodium hydroxide solution to remove any acetyl groups. The guaran solution was then acidified to pH 6 with hydrochloric acid and was precipitated with 1-1/2 volumes of methanol to remove any borax. The guaran was collected by filtration, dissolved in water, thoroughly deionized with Amberlite MB-3 (H^+, OH^-), concentrated, and freeze dried.

A sample of purified guar gum, 3.82 g. o.d., was treated in a two-stage oxidation similar to that used in oxidizing galactoglucomannan. However, the guaran was oxidized just once; and the experimental conditions are shown in Table VIII.

The recovery of oxidized guar gum was approximately 97%. Small samples, about 100 mg., of oxidized and unoxidized guar gum were sent to the Analytical Section for sugar (28) and uronic acid (Institute Method 25) analyses. The results are shown in Table IX.

TABLE VIII

EXPERIMENTAL OXIDATION CONDITIONS

STAGE 1:	
Temperature, °C.	30 ± 1
Time of enzymatic oxidation, hours	4
Concentration of galactose residue, mg./ml.	0.5
Concentration of galactose oxidase, units/mg. galactose	1.5
Concentration of catalase, units/mg. galactose	1400
Concentration of pH 7 buffer, ml./mg. galactose	0.7
STAGE 2:	
Temperature, °C.	30 ± 1
Time of iodometric oxidation, hours	4
Concentration of galactose residues, mg./ml.	0.2
Concentration of iodine, mg./mg. galactose	10
Concentration of sodium carbonate, mg./mg. galactose	13

TABLE IX

ANALYSES OF OXIDIZED AND UNOXIDIZED GUARAN

	Guaran	Oxidized Guaran
Mannan, %	59.0	52.9
Galactan, %	39.6	24.1
Uronic acid, %[a]	<1.0	21.0
Total, %	98.6	98.0

[a] Carbon dioxide evolution calculated as uronic acid.

PRELIMINARY ACID HYDROLYSIS EXPERIMENTS

ACID HYDROLYSIS OF OXIDIZED GALACTOGLUCOMANNAN

Approximately 30 mg. of thrice-oxidized galactoglucomannan were subjected to a partial acid hydrolysis. The oxidized polymer was dissolved in 20 ml. of 2N sulfuric acid and heated at 90 ± 3°C. for six hours. In order to remove the sulfate ion the hydrolysate was neutralized with barium carbonate and vacuum-filtered through a sintered-glass funnel covered with a cellulose pad. After deionization with Amberlite IR-120 (H^+), the hydrolysate was evaporated to dryness on a rotary evaporator and placed in a vacuum desiccator.

In order to observe the presence of any acidic compounds other than galacturonic acid, the hydrolysate was chromatographed in two dimensions using descending solvent systems. The hydrolysate of oxidized galactoglucomannan was dissolved in distilled water, and approximately 2 mg. were spotted on a sheet of Whatman No. 1 chromatography paper. The chromatogram was developed for three days in Solvent A, which moves both neutral and acidic compounds. There was little chance of losing acidic dimers containing galacturonic acid since this acid does not migrate off a sheet of Whatman No. 1 after developing for two days in Solvent A. After air drying, the chromatogram was placed in Solvent B which moves only neutral compounds and developed for two days in a direction perpendicular to the flow of Solvent A. After treatment with Solvent B, the chromatogram was air dried; and the migrating compounds were detected with silver nitrate dip. A series of spots was detected which was unmoved by the basic solvent system, Solvent B; and they were called A1-A4. These spots are probably acidic compounds, and a diagrammatic representation of the chromatogram is shown in Fig. 7.

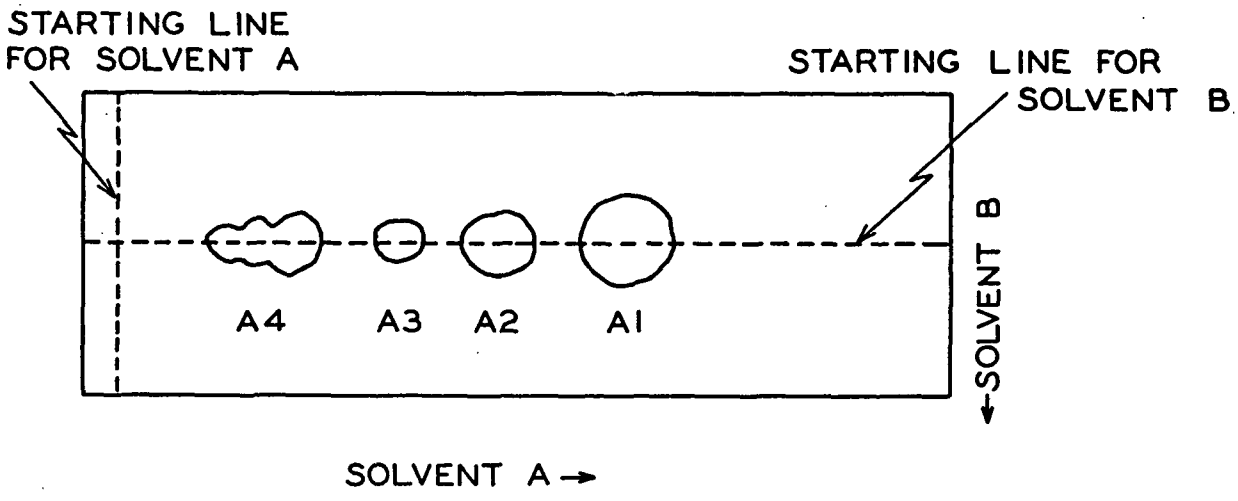

Figure 7. Chromatogram of Hydrolysate from Oxidized Galactoglucomannan

ACID HYDROLYSIS OF UNOXIDIZED GALACTOGLUCOMANNAN

For control purposes, about 30 mg. of unoxidized polymer were hydrolyzed and chromatographed in exactly the same manner as the oxidized polymer. Chromatographic evidence of acidic material in the hydrolysate of the unoxidized galactoglucomannan was found. The major component migrated at the same rate as A2 in Solvent A and, like A2, was unmoved by Solvent B. Some very light streaking was also found which was not moved by Solvent B; this material migrated at approximately the rate of A1 in Solvent A.

In another experiment the unoxidized galactoglucomannan hydrolysate was spotted on a sheet (7-1/2 inches x 18 inches) of Whatman No. 3 chromatography paper (all Whatman No. 3 paper was washed for two days with a 1:1 mixture of ethanol and water). The chromatogram was placed in Solvent A for three days followed by development in Solvent B for three days. A guide strip was cut from the chromatogram and dipped in silver nitrate to locate the migrating components, and the section of the chromatogram believed to contain the major acidic component was removed and eluted with water. After determining when elution was complete utilizing an anthrone-sulfuric acid reagent (35), the eluate was evaporated to dryness on a rotary evaporator.

The residue was dissolved in 2 ml. of 4.5% sulfuric acid and heated for 45 minutes in a household pressure cooker at 120°C. In order to remove the sulfate ion, the hydrolysate was neutralized with barium carbonate and vacuum-filtered through a sintered-glass funnel with a cellulose mat. After deionization with Amberlite IR-120 (H^+) and evaporation to dryness, approximately 25 μg. of hydrolysate were spotted along with controls (known compounds) on a sheet of Whatman No. 1 chromatography paper. The chromatogram was developed in Solvent C for 24 hours followed by development in Solvent B for another 24 hours, and silver nitrate dip was used to locate the various compounds. The hydrolysate was resolved into components which moved at the same rate as glucuronic acid, galactose, glucose, and xylose, but no compound migrating at the same rate as galacturonic acid was observed.

ACID HYDROLYSIS OF OXIDIZED AND UNOXIDIZED GUARAN

Samples of oxidized and unoxidized guaran, approximately 30 mg., were hydrolyzed and examined with two-dimensional chromatography under the same conditions as the oxidized galactoglucomannan. Although no evidence of acidic material was found in the hydrolysate of unoxidized guaran, acidic compounds were found in the hydrolysate of oxidized polymer. The major component that was unmoved by Solvent B had the same mobility in Solvent A as the A1 component from oxidized galactoglucomannan. Evidence was also obtained for small amounts of higher molecular weight acidic compounds from oxidized guaran.

ISOLATION OF ACIDIC COMPONENTS

ACID HYDROLYSIS OF OXIDIZED GALACTOGLUCOMANNAN AND OXIDIZED GUARAN

A thrice-oxidized sample of galactoglucomannan, 3.39 g. o.d.*, was dissolved in 1.8 liters of distilled water; the polymer solution was heated in an autoclave

*Corrected for retained enzyme.

at 15 p.s.i. for 25 minutes as were 1.8 liters of 4N sulfuric acid. The two solutions were then mixed together and heated at 90 ± 3°C. for three hours at atmospheric pressure. The sulfate ion was removed from the hydrolysate by neutralization with barium carbonate and vacuum-filtration through a coarse, sintered-glass funnel covered with a cellulose pad. The hydrolysate was deionized with Amberlite IR-120 (H^+) and concentrated to a sirup on a rotary evaporator. Preparative chromatograms were prepared by spotting the sirup on 15 sheets of Whatman No. 3 chromatography paper.

The oxidized guaran, 1.98 o.d.*, was dissolved in 1 liter of distilled water; the solution was heated in an autoclave at 15 p.s.i. for 25 minutes as was 1 liter of 4N sulfuric acid. The two solutions were then mixed together and heated at 90 ± 3°C. for three hours. The treatment of the oxidized guaran hydrolysate was identical to that of the oxidized galactoglucomannan hydrolysate described in the previous paragraph. The concentrated hydrolysate was spotted on nine sheets of Whatman No. 3 chromatography paper.

PREPARATIVE CHROMATOGRAPHY

The preparative chromatograms containing the hydrolysates of oxidized galactoglucomannan and oxidized guaran were developed for two weeks in Solvent B. Guide strips were cut from representative chromatograms at various times and the migrating compounds were detected with silver nitrate dip. These guide strips indicated that after two weeks the neutral compounds were well separated from the acidic material which was still on the starting line. The preparative chromatograms were removed from the basic solvent tank, air dried, and developed with Solvent A for one week to separate the acidic compounds. After removing the chromatograms from Solvent A and air drying, two guide strips were cut from each chromatogram; and silver nitrate dip was used to locate the various compounds.

*Corrected for retained enzyme.

The guide strips from the chromatograms containing the hydrolysate of oxidized galactoglucomannan showed six distinct spots. In the preliminary hydrolysis experiments of this polymer, four spots were unmoved by Solvent B. However, component A4 from the preliminary hydrolysis experiments was resolved into three components when developed with Solvent A for one week. The six spots were designated A1-A6 with the fastest and slowest being A1 and A6, respectively. The preparative chromatograms were cut into the appropriate sections, corresponding to A1-A6; and the acidic materials were eluted with distilled water.

The guide strips from the oxidized-guaran chromatograms showed one distinct spot and some evidence (streaking) of higher molecular weight materials. The major component was called A and had the same mobility in Solvent A as component A1 from the hydrolysate of oxidized galactoglucomannan. The sections containing Component A were cut from the preparative chromatograms and eluted with distilled water.

In order to determine when elution was complete, the eluates were tested with an anthrone-sulfuric acid reagent (35). The eluates were deionized with Amberlite IR-120 (H^+), evaporated to dryness on a rotary evaporator, and stored in a vacuum desiccator. The yields of the various components and their relative mobilities, $R_{\underline{x}}$*, are given in Table X; and a diagrammatic representation of a chromatogram containing the acidic materials is shown in Fig. 8.

IDENTIFICATION OF ACIDIC COMPONENTS

The low yields of the acidic materials prohibited a rigorous characterization by methylation and identification of hydrolysis products. Therefore, other experimental means for characterizing these acidic materials were investigated.

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*$R_{\underline{x}}$ = Distance traveled in cm./xylose distance in cm.

TABLE X

YIELDS AND MOBILITIES OF ACIDIC COMPONENTS

Component	Yield, mg.	Yield, % of oxidized polymer[a]	R_x
A	45	2.3	0.17
A1	50	1.5	0.17
A2	30	0.89	0.13
A3	9	0.27	0.11
A4	11	0.32	0.082
A5	5	0.15	0.064
A6	3	0.09	0.055
Xylose	--	--	1.00

[a]Corrected for retained enzyme.

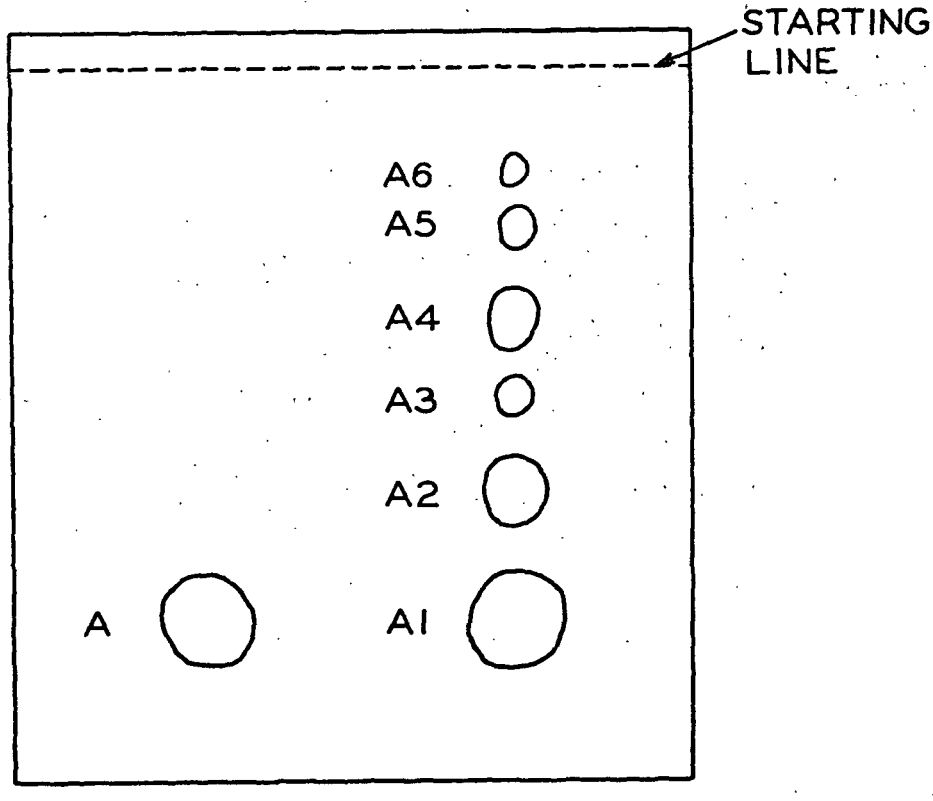

Figure 8. Hydrolysates of Oxidized Galactoglucomannan and Guaran

SUGAR RATIOS BY QUANTITATIVE PAPER CHROMATOGRAPHY

The ratios of the monomeric sugars in the acidic components were determined using a modification of the spot area technique described by Fisher, et al. (36). This technique is based on the fact that many compounds react with indicators to give spot areas which are linearly related to the logarithms of the spot contents. Care must be exercised when using this procedure since at very high concentrations (spot contents) this relationship breaks down (37).

Approximately 1-2 mg. of each acidic material and an authentic sample of 6-O-β-D-glucopyranosyluronic acid-D-galactopyranose was hydrolyzed with 2 ml. of 4.5% sulfuric acid for one hour in a household pressure cooker at 120°C. The hydrolysates were neutralized with barium carbonate, vacuum-filtered through a coarse, sintered-glass funnel with cellulose pad, deionized with Amberlite IR-120 (H^+), and evaporated to dryness on a rotary evaporator. Duplicate spot-area chromatograms were prepared for each hydrolysate using Whatman No. 1 chromatography paper; and they were spotted, using a Gilmont ultramicroburet, in five different locations with known compounds and in two different locations with unknown (hydrolysate). On each chromatogram the knowns were spotted with increasing concentration from 0.5 to 3.5 μg., and the unknowns were spotted in two different concentrations within the limits set by the knowns.

The spot-area chromatograms were developed for 24 hours with Solvent C; and after air drying, they were developed with Solvent B for another 24 hours. The migrating compounds were detected by dipping the chromatograms in silver nitrate indicator, and the spot areas were outlined by placing the chromatograms on a lighted, translucent glass plate and tracing the darkened sections. After tracing the areas onto graph paper, a measure of the relative areas was obtained by cutting the areas from the graph paper and weighing them on an analytical balance. Since

the basis weight variation of the graph paper was negligible, this proved to be a fast and accurate method for determining relative spot areas.

The areas of the knowns were plotted against the spot contents, and straight lines were drawn through the data points, as shown in Fig. 9. Knowing the spot areas of the unknowns, the spot contents were read off the curves. Chromatographic evidence for various monomeric compounds was found in each hydrolysate and the ratios of these compounds are given in Table XI. Acidic component A6 showed evidence of glucose and smaller amounts of galacturonic acid and mannose. However, the spot areas were too small to analyze and this material was not investigated further.

TABLE XI

SUGAR RATIOS

Component	Compounds	Sugar Ratio Observed	Corrected[a]
A	galacturonic acid: mannose	0.7:1	1:1
A1	galacturonic acid: mannose	0.7:1	1:1
A2	glucuronic acid: galactose: galacturonic acid: glucose	(-)[c]:0.5:0.7:1	(-)[c]:0.5:1:1
A3	galacturonic acid: galactose	0.7:1	1:1
A4	galacturonic acid: glucose: mannose	0.7:1:0.9	1:1:0.9
A5	galacturonic acid: glucose: mannose	0.7:0.9:1	1:0.9:1
A6	galacturonic acid: glucose: mannose	--	--
X[b]	glucuronic acid: galactose	0.7:1	1:1

[a] Based on values obtained for Component X.

[b] 6-O-β-D-Glucopyranosyluronic acid-D-galactopyranose.

[c] Spot area too small to measure.

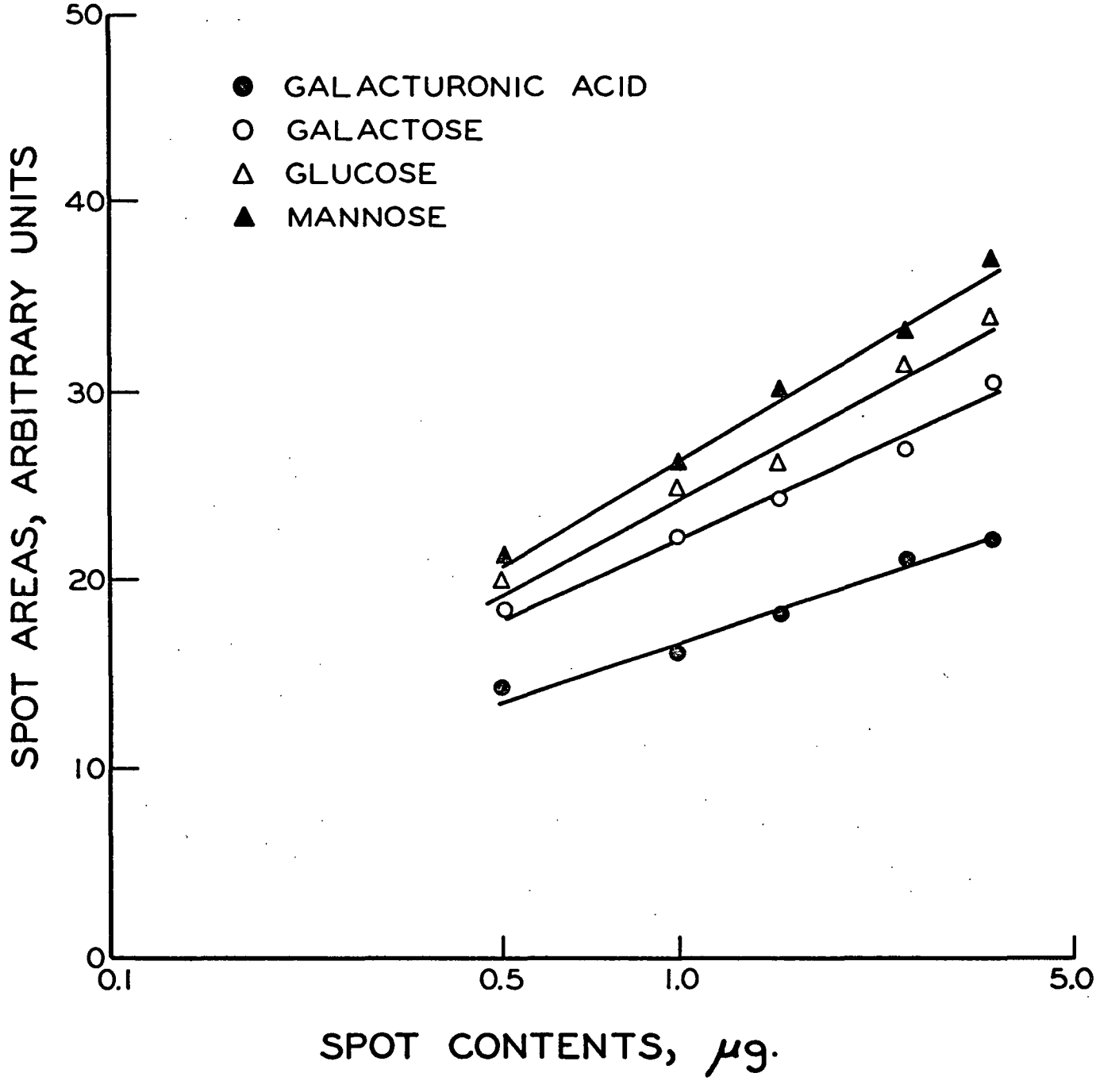

Figure 9. Typical Spot-Area Plot

SODIUM BOROHYDRIDE REDUCTION

In order to elucidate the sequence of linkages, the acidic materials were reduced with sodium borohydride by a modification of the technique reported by Abdel-Akher, et al. (38). Approximately 1-2 mg. of each acidic material was dissolved in 10 ml. of water to which was added 5 ml. of freshly prepared 1% sodium borohydride solution. After reduction for one hour at ambient temperature, the excess borohydride was destroyed by addition of glacial acetic acid until the evolution of hydrogen ceased. After the solutions were deionized with Amberlite IR-120 (H^+) and evaporated to dryness on a rotary evaporator, each sample contained a large amount of white, powdery residue (boric acid). This was removed as the volatile methyl borate by making 3-10 ml. additions of methyl alcohol to each sample with evaporation to dryness after each addition.

HYDROLYSIS OF REDUCTION PRODUCTS

The reduced samples, RA, RA1-RA3, were dissolved in 2 ml. of 4.5% sulfuric acid and hydrolyzed for one hour in a household pressure cooker at 120°C. The sugar ratio data can be interpreted to indicate that A4 and A5 are aldotriouronic acids. In an attempt to obtain evidence of aldobiouronic acids, the reduced components RA4 and RA5 were hydrolyzed for one-half hour; the other conditions remained the same. All of the hydrolysates were neutralized with barium carbonate, vacuum-filtered through a sintered-glass funnel with cellulose pad, deionized with Amberlite IR-120 (H^+), and evaporated to dryness on a rotary evaporator. Two chromatograms were prepared for each hydrolysate by spotting sheets of Whatman No. 1 chromatography paper with approximately 25 µg. of hydrolysate as well as known compounds. The chromatograms were developed for 24 hours in Solvent C followed by development in Solvent B for another 24 hours.

One of the two chromatograms of each hydrolysate was treated with silver nitrate dip while the others were sprayed with p-anisidine hydrochloride. Silver nitrate dip detects both reducing and nonreducing sugars while p-ansidine hydrochloride detects only reducing sugars. By analyzing the two chromatograms from each hydrolysate, the reducing end-groups in each acidic material were determined. The compounds from the hydrolysates which were indicated by the two detectors are shown in Table XII.

TABLE XII

REDUCTION DATA

Component	Silver Nitrate	p-Anisidine Hydrochloride
RA	galacturonic acid, mannitol	galacturonic acid
RA1	galacturonic acid, mannitol	galacturonic acid
RA2	galacturonic acid, glucuronic acid, galactitol, glucitol	galacturonic acid, glucuronic acid
RA3	galacturonic acid, galactitol	galacturonic acid
RA4	galacturonic acid, glucose, mannitol	galacturonic acid, glucose
RA5	galacturonic acid, glucose mannose, glucitol, mannitol	galacturonic acid, glucose, mannose

Approximately 50-µg. samples of the hydrolysates from RA4 and RA5 were spotted on a sheet of Whatman No. 1 chromatography paper along with about 25 µg. of A1 and A2. After developing for one week in Solvent A, the chromatogram was treated with silver nitrate dip, and the major component from the hydrolysate of RA4, which was detected, migrated at the same rate as A2. It was found that two major components from the hydrolysate of RA5 were detected; one migrated at the same rate as A1 while the other had the mobility of A2.

The remainder of the hydrolysate of RA4 was spotted on a sheet of Whatman No. 3 chromatography paper and developed in Solvent A for one week. A guide strip was cut from the chromatogram and dipped in silver nitrate to locate the migrating compounds; the section containing the compound with the same mobility as A2 was removed and eluted with distilled water. The eluate was evaporated to dryness, and the residue was dissolved in 1 ml. of 4.5% sulfuric acid and heated for 45 minutes in a household pressure cooker at 120°C. The hydrolysate was neutralized with barium carbonate, vacuum-filtered on a sintered-glass funnel with cellulose pad, deionized with Amberlite IR-120 (H^+), and concentrated to a sirup on a rotary evaporator. Approximately 10 µg. of the hydrolysate were spotted along with knowns on a sheet of Whatman No. 1 chromatography paper, and it was developed for 24 hours in Solvent C followed by 24 hours in Solvent B. The chromatogram was then dipped in silver nitrate, and compounds migrating at the same rate as galacturonic acid and glucose were observed.

IDENTIFICATION OF D-GALACTURONIC ACID

In order to obtain a suitable quantity of acidic material for the formation of a derivative, approximately 13 mg. of A1 were hydrolyzed. The sample of A1 was dissolved in 4 ml. of 4.5% sulfuric acid and heated for 45 minutes in a household pressure cooker at 120°C. The hydrolysate was neutralized with barium carbonate, vacuum-filtered on a sintered-glass funnel with cellulose mat, deionized with Amberlite IR-120 (H^+), and concentrated to a sirup on a rotary evaporator. The sirup was spotted on a sheet of Whatman No. 3 chromatography paper, and the chromatogram was developed for 24 hours in Solvent A followed by treatment with Solvent B for 24 hours. A guide strip was removed, and the migrating compounds were detected with silver nitrate dip. The section containing the suspected D-galacturonic acid was cut from the chromatogram and eluted with distilled water; the eluate was then evaporated to dryness on a rotary evaporator.

The "D-galacturonic acid" was proved to be authentic by conversion to a derivative, mucic acid. The suspected D-galacturonic acid, approximately 4-5 mg., was dissolved in 10 ml. of saturated bromine water; and the solution was kept for two hours at room temperature. After two hours, the solution was placed in an exhausting hood for 1-1/2 days; after which time the solution was evaporated to dryness by directing a stream of dry air on its surface. The crystalline residue contained a reddish-brown tar which was removed by washing with several drops of 95% ethanol. The x-ray diffraction pattern of the white, water-insoluble crystalline material was determined; and the resulting pattern is compared to the pattern of an authentic sample of mucic acid in Fig. 10.

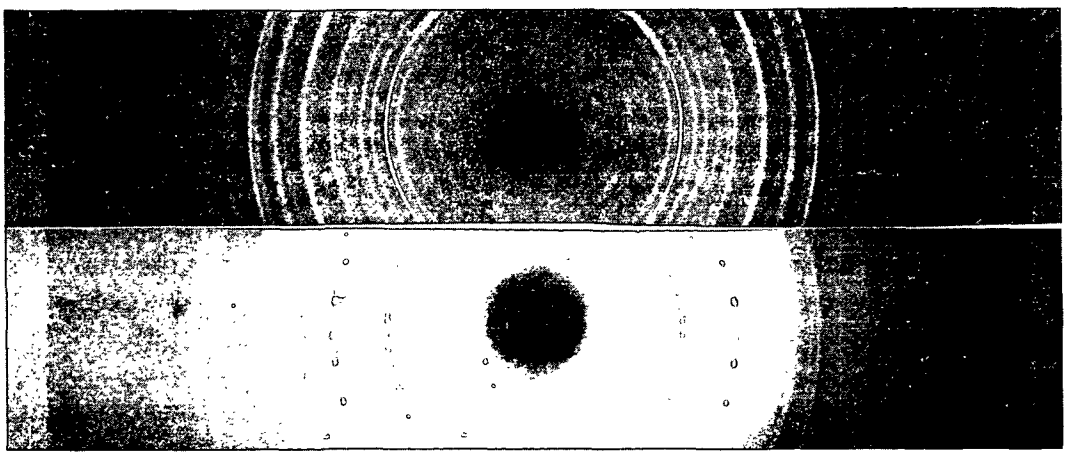

Figure 10. X-ray Patterns of Mucic Acid (Upper) and Suspected Mucic Acid (Lower)

EQUIVALENT WEIGHTS

The quantities of A and Al were sufficient for analysis of equivalent weights, but since these materials were a light brown color, they were first decolorized with charcoal. Each compound, approximately 30 mg., was dissolved in 20 ml. of distilled water to which was added 30 mg. of Darco G-60* which had been washed three times

*Darco G-60 is a vegetable, charcoal powder obtained from E. H. Sargent and Company.

each with hot and cold distilled water. After standing for 30 minutes with frequent agitation, the mixtures were vacuum-filtered through a Celite*-covered cellulose mat which had been washed three times each with hot and cold distilled water. The filtrates were evaporated to dryness on a rotary evaporator and stored in a vacuum desiccator; the decolorized compounds were an off-white color.

The equivalent weights were evaluated by a modification of the procedure described by Steyermark (39). The sample, 4-5 mg. o.d., was dissolved in hot distilled water; and one drop of phenolphthalein indicator was added before the acidic solution was titrated with 0.106N sodium hydroxide using a Gilmont ultra-microburet. The reaction vessel was continually swept with a stream of nitrogen, and the solution was mixed by bubbling nitrogen through it; the end point was reached when the characteristic pink color persisted one minute. Duplicate determinations were made on each compound, and the equivalent weights were 411 for A and 380 for A1. Since these materials were amorphous, the values compare favorably with the theoretical equivalent weight of 356 for O-D-galactopyranosyl-uronic acid-D-mannopyranose.

INFRARED SPECTRA

The infrared spectra of Compounds A and A1 were determined, and they are shown in Fig. 11. The shape of the curves confirms the speculation that these acids have identical structures although isolated from different sources.

SPECIFIC ROTATIONS

The specific rotations, $[\alpha]_D^{29}$(water), of A and A1 were measured. The specific rotation of A was +111 while that of A1 was +101; these values indicate that the glycosidic linkages are of the α-configuration. The specific rotation, $[\alpha]_D^{29}$(water), of A2 was also determined and found to be +65.

*Celite is an analytical filter-aid produced by Johns-Manville.

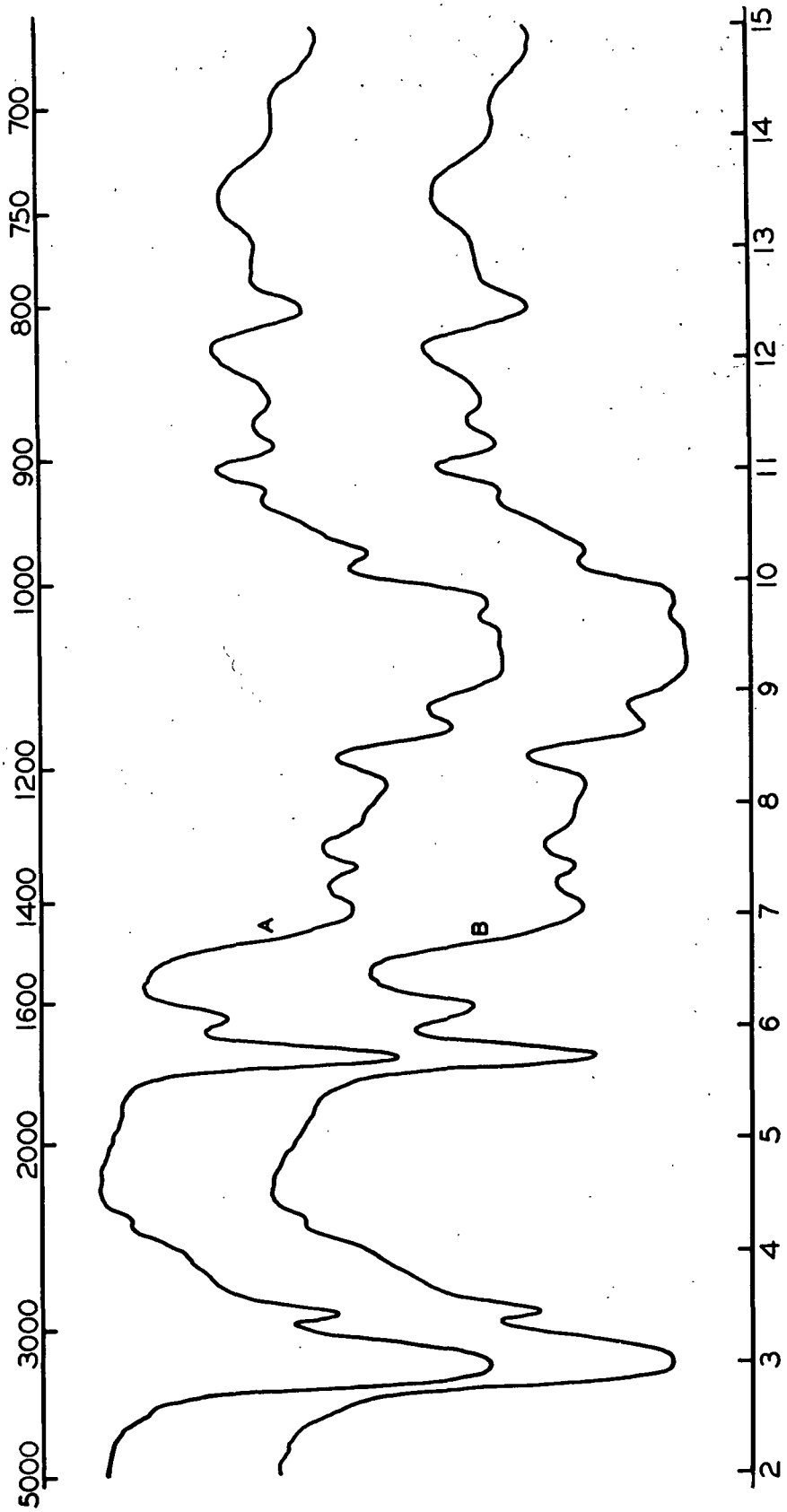

Figure 11. Infrared Spectra of A (Curve B) and A1 (Curve A)

DISCUSSION OF RESULTS AND CONCLUSIONS

PROPERTIES OF GALACTOGLUCOMANNAN

The most common method of isolating galactoglucomannan polymers is by the potassium-hydroxide extraction of chlorite holocelluloses. Mannose-rich polymers obtained in this way are generally purified quite well by several barium hydroxide precipitations (4); the barium ions form an insoluble complex with the polymer, probably by reaction with the vicinal cis-hydroxyl groups on Carbon Atoms 2 and 3 of the mannose units (40). Recently, it has been reported that when the chlorite holocelluloses of conifers are prepared, some galactoglucomannan dissolves in the liquor (1, 2). The galactoglucomannan polymer used in this investigation was isolated from the chlorite liquor and was purified by precipitation with barium hydroxide and with Fehling solution. The results of free-boundary electrophoresis on the purified polymer preparation indicate the presence of a galactan contaminant as well as minor amounts of xylose and arabinose contamination. Although the polymer preparation could have been purified further by free-boundary electrophoresis, no attempt was made to do so because of the prohibitive time factor involved. Although not thoroughly investigated, it appears feasible that the difficulty encountered in purifying the polymer is inherent in galactoglucomannan preparations isolated from chlorite liquors.

The data in Table IV, p. 17, indicate that approximately 15% of the galactoglucomannan preparation is not accounted for by either sugars, uronic acid, or ash. Since the chloriting procedure destroys some of the aromatic residues in lignin (41), the data from the alkaline nitrobenzene oxidation of galactoglucomannan demonstrates a lesser amount of lignin (1.8%) than is actually present. The presence of acetone-soluble material in the galactoglucomannan preparation is further evidence of lignin debris since it is doubtful that carbohydrate material would be

soluble. Croon, et al. (10) and Linnell (29) isolated and purified glucomannan preparations and found that the polymers contained lignin contamination which they proposed was difficult to remove because of a lignin-carbohydrate linkage. Therefore, it was concluded that much of the remainder of the polymer preparation was lignin debris.

The hydrolysate of the polymer preparation showed little or no chromatographic evidence of uronic acids; however, the polymer had a spuriously high "uronic acid" content as seen in Table IV, p. 17. A similar phenomenon has been observed by Thompson, et al. (42) in an investigation of a galactoglucomannan isolated from softwood kraft pulp. They found that the polymer preparation contained an apparent uronic acid content of more than 4% when analyzed by conventional carbon dioxide evolution, although no uronic acid was observed on paper chromatography. These occurrences are at least partially explained by the investigation of Milks and Purves (43). They determined that the apparent uronic acid content of a spruce periodate lignin was 8.4% before and 21.3% after oxidation with chlorine dioxide. Therefore, it is proposed that most of the "uronic acid" in the present investigation originated from oxidized lignin that remained in the galactoglucomannan preparation.

OXIDIZED GALACTOGLUCOMANNAN AND GUARAN

One of the objectives of this study was the formation of galacturonides from the galactoglucomannan and guaran polymers, and the experimental results show that this was accomplished. First of all, Avigad, et al. (19) demonstrated that galactose oxidase has little or no effect on glucose or mannose residues; and this was confirmed in the present investigation. The analyses of D-galactose and uronic acid indicate that D-galactose groups were converted to D-galacturonic acid residues by the two-stage oxidations, see Table VII, p. 23 and Table IX, p. 24. Also, the suspected D-galacturonic acid had the same mobility as an authentic sample in all

three solvent systems but different from D-glucuronic acid in Solvents A and C and D-mannuronic acid in Solvent A (the only one tested). Finally, the conversion of suspected D-galacturonic acid to mucic acid demonstrates unequivocally that D-galacturonic acid residues were formed by oxidation of the galactoglucomannan and guaran polymers.

The present investigation is the first reported oxidation of hemicelluloses with galactose oxidase and iodine solution, and it is also the first time a galacturono-galactoglucomannan and a galacturono-galactomannan have been prepared. The procedure used in this investigation is a new method for studying the structural aspects of polysaccharides and is probably applicable to all polymers containing D-galactose with unblocked C6 positions.

SPECTRUM OF ACIDIC COMPONENTS

The second objective of the present study was the isolation and characterization of the acidic compounds obtained from the partial acid hydrolysis of the oxidized galactoglucomannan and guaran. The acidic components were characterized within the limits of available material, and the results are discussed below. It was observed that a trace amount of xylose impurity was present in the hydrolysate of each component.

SUGAR RATIOS

If a heterodimer is hydrolyzed with acid to its monomeric units and each one is degraded to the same extent, a sugar ratio of 1:1 would be expected. Whistler, et al. (44) have shown that under acid hydrolysis conditions uronic acid monomers are destroyed more rapidly than neutral monomers. Therefore, when an aldobiouronic acid is hydrolyzed with mineral acid the amount of uronic acid remaining after hydrolysis will be less than that of the neutral compound. In order to investigate

the effect of acid hydrolysis on aldobiouronic acids more closely, a known sample of crystalline 6-O-β-D-glucopyranosyluronic acid-D-galactopyranose was hydrolyzed. The sugar ratio was determined by the spot-area technique, and the ratio of D-glucuronic acid to D-galactose was 0.7:1, Table XI, p. 32. By analogy, it appears that the sugar ratios in Table X represent dimers and trimers.

COMPONENTS A AND A1

When the sugar-ratio, reduction, and equivalent weight data are considered, it is quite clear that Compounds A and A1 are aldobiouronic acids with mannose as the terminal reducing residue. The infrared spectra of these compounds indicate that they are identical, and the specific rotations suggest glycosidic linkages of the α-configuration.

The guaran polymer is a straight-chain mannan with single member D-galactopyranose side chains which are attached to the backbone by α-1,6-linkages (22, 23). Since Compound A was obtained from oxidized guaran, it is very probable that the glycosidic linkage is α-1,6. The previous structural investigations of galactoglucomannan indicate that single D-galactopyranose units are attached to mannose of the backbone by α-1,6-linkages (4) which implies that the glycosidic linkage in A1 is α-1,6. A number of compounds containing one D-galactose unit and D-mannose residue(s) have been isolated from the hydrolysates of galactoglucomannans, and in each instance the galactose was linked to the mannose by an α-1,6-linkage (1, 9). On the basis of the previous evidence, it is concluded that A and A1 represent a new aldobiouronic acid, 6-O-α-D-galactopyranosyluronic acid-D-mannopyranose.

COMPONENT A2

Past structural studies of galactoglucomannan polymers indicate that the D-galactose residues terminating the branches attached to the D-glucose units of

the backbone are joined by α-linkages (4). Therefore, the partial acid hydrolysis of oxidized galactoglucomannan would be expected to yield an acidic dimer, O-α-D-galactopyranosyluronic acid-D-glucopyranose. The sugar-ratio and reduction data demonstrate that Component A2 is composed primarily of D-galacturonic acid and D-glucose with lesser amounts of D-glucuronic acid and D-galactose. However, it is not surprising that A2 is a mixture of compounds since the hydrolysate of unoxidized galactoglucomannan contained an acidic component with the same mobility as A2 in Solvent A. When this acidic material from the unoxidized polymer was hydrolyzed, chromatographic evidence of glucuronic acid was obtained.

The sugar-ratio and reduction data can be interpreted to indicate that Component A2 is made up of two parts D-galactopyranosyluronic acid-D-glucopyranose and one part D-glucopyranosyluronic acid-D-galactopyranose. While conducting an investigation of the less-abundant polysaccharides in conifers, Thompson and Kaustinen (2) isolated and identified an aldobiouronic acid, 6-O-β-D-glucopyranosyluronic acid-D-galactopyranose, from the hydrolysate of a component from black spruce chlorite liquors. An authentic sample of this disaccharide acid has the same mobility as A2 in Solvent A which suggests that it might be the contaminant in Component A2.

The specific rotation, $[\alpha]_D^{29}$(water), of A2 was found to be +65; and the specific rotation, $[\alpha]_D^{32}$(water), of 6-O-β-D-glucopyranosyluronic acid-D-galactopyranose (X) has been reported as -6.2 (c 1. water) (2). Assuming A2 is two thirds O-D-galactopyranosyluronic acid-D-glucopyranose and one third X, as the sugar-ratio data implies, the specific rotation of the former compound is calculated as +101 which is indicative of an α-linkage.

It is significant that Component A2 demonstrates for the first time the presence of a galactose-glucose linkage in galactoglucomannan polymers. The experimental evidence strongly indicates that the major acidic material in Component A2 is another

new aldobiouronic acid, O-α-D-galactopyranosyluronic acid-D-glucopyranose. Assuming the D-galactose groups attached to the D-glucose residues are single member side chains, the glycosidic linkage is α-1,6; if not, the linkage is α-1,4 (see Fig. 3, p. 6).

COMPONENT A3

The sugar-ratio and reduction data indicate that Component A3 is an aldobiouronic acid, O-D-galactopyranosyluronic acid-D-galactopyranose, with galactose as the reducing unit. The majority of the previous structural studies of galactoglucomannans gave no evidence of a galactose-galactose linkage (4). However, in one structural investigation of this polymer, galactobiose and galactotriose were isolated but were attributed to a galactan contaminant (1). Since, in the present investigation, the free-boundary electrophoresis data indicate the presence of a galactan impurity in the polymer preparation, it is concluded that Component A3 derives from the galactan contaminant.

COMPONENT A4

The experimental evidence, sugar-ratio and reduction data, indicates that Component A4 is an aldotriouronic acid with mannose as the reducing residue; and since the partial acid hydrolysis of RA4 yielded a component, composed of D-galacturonic acid and D-glucose, with the same mobility as A2 in Solvent A, the sequence of linkages is inferred. The experimental results indicate that the structure of A4 is: O-α-D-galactopyranosyluronic acid-O-D-glucopyranosyl-D-mannopyranose which is additional evidence for the existence of a galactose-glucose linkage in galactoglucomannans. Although Mills and Timell (8) isolated a triheterotrimer from a galactoglucomannan, the compound was not characterized. The isolation and partial characterization of A4, a unique aldotriouronic acid, is strong inference for the existence of a triheteropolymer galactoglucomannan.

COMPONENT A5

According to the sugar-ratio and reduction results, Component A5 is a mixture of compounds, most probably aldotriouronic acids. Since the partial acid hydrolysis of RA5 yields components which have the same mobilities as A1 and A2 in Solvent A, the sequence of sugar residues is partially fixed; however, the specific nature of Component A5 is unknown.

HYPOTHESIS

It will be recalled that the hypothesis employed in this investigation was that a true triheteropolymer galactoglucomannan exists in the wood of gymnosperms with some of the D-galactose groups attached directly to the D-glucose residues. Strong support for this hypothesis was obtained in the present investigation by Component A2 which demonstrates the existence of a galactose-glucose bond of the α-configuration. Component A4 is also very significant since it is a strong argument for the existence of a triheteropolymer galactoglucomannan as well as the existence of a galactose-glucose linkage.

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APPENDIX I

ISOLATION OF A GALACTOGLUCOMANNAN FROM THE HOLOCELLULOSE

The total o.d. weight of holocellulose was 6780 g., and four batches of holocellulose were extracted with 24% potassium hydroxide at 10% consistency for two hours at room temperature. After each batch extraction the holocellulose was filtered using two large, sintered-glass funnels; and each filterful was washed with 1 liter of deionized water. The extract liquor and wash water were combined and placed in a 24-liter battery jar; the total amount of liquid from one batch extraction was about 12 liters. This liquor was made 5% (w:v) in sodium chlorite, acidified to pH 6 with glacial acetic acid, and retained overnight to degrade the lignin debris. Each of the four extractions was carried out as indicated above.

To each batch of potassium hydroxide extract liquor was added about 2000 g. of sodium hydroxide and approximately 900 g. of barium acetate; this caused complete precipitation of the barium-complex which was separated and collected by centrifugation. The barium precipitates were combined and made into a paste with deionized water, and the pH was adjusted to 6 with glacial acetic acid. Most of the precipitate went into solution, and the insoluble material was removed by centrifugation. Approximately 400 g. of sodium hydroxide were added to the supernatant, and the second barium precipitate was immediately formed and collected by centrifugation. It was made into a paste with deionized water, acidified to pH 6 with glacial acetic acid, and centrifuged to remove the insoluble material. The supernatant was made alkaline with 500 g. of sodium hydroxide; the third barium precipitate formed immediately and was collected by centrifugation. It was treated the same as the second barium precipitate.

The galactoglucomannan was purified further by precipitation with an equal volume of freshly prepared Fehling solution and was collected by centrifugation.

The precipitate was made into a paste with deionized water, acidified to pH 6 with glacial acetic acid, and centrifuged to remove the insoluble material. After dialyzing the supernatant against deionized water for six days to remove most of the copper ions, the polymer solution was removed from the dialysis sacks and concentrated to 5 liters using two cyclone evaporators. The concentrated polymer was deionized with Amberlite IR-120 (H^+) to remove the remaining copper ions and filtered through a Celite-covered cellulose pad on a sintered-glass funnel. The 6 liters of filtrate were concentrated to 3 liters on a cyclone evaporator; and the clear, amber polymer solution was freeze dried.

The yield of o.d. galactoglucomannan was approximately 140 g. which represents about 1.5% of the o.d. wood. Quantitative sugar analyses were conducted on the polymer using the method of Saeman, et al. (28), and uronic acid was determined according to Institute Method 25. The properties of the galactoglucomannan are shown in Table XIII.

TABLE XIII

PROPERTIES OF GALACTOGLUCOMANNAN[a]

Glucan, %	19.2
Mannan, %	57.8
Galactan, %	6.68
Xylan, %	3.74
Araban, %	0.47
Uronic acid, %[b]	9.32
Ash, % (27)	1.25
Total, %	98.5

[a] Timell's extraction procedure was employed; however, the molar ratio of galactose, glucose, and mannose was 0.3:1:3 compared to the ratio of 1:1:3 reported previously (4).

[b] Carbon dioxide evolution calculated as uronic acid.

APPENDIX II

PRELIMINARY EXPERIMENTS

ENZYME OXIDATION OF GALACTOSE, GLUCOSE, MANNOSE, AND GALACTOGLUCOMANNAN

The action of galactose oxidase on galactose, glucose, mannose, and galactoglucomannan was investigated. There were samples number 1, 2, and 3 for each reaction time, and the reagents used in the different samples are given in Table XIV. After the desired reaction time, the duplicate samples were treated with 6 ml. of 0.02N iodine solution and 0.4 ml. of 5% sodium carbonate solution. The reaction flasks were kept in the constant temperature bath an additional 15 minutes before 1 ml. of 0.5N sulfuric acid was added to each one. The oxidizing power in the reaction vessels was then determined by titrating with 0.005N sodium thiosulfate. The meq. of thiosulfate needed to reduce a mixture of 6 ml. of 0.02N iodine solution, 0.4 ml. of 5% sodium carbonate solution, and 1 ml. of 0.5N sulfuric acid was also determined.

The samples numbered 1 indicate how much iodine was reduced by the unoxidized (by enzyme) sugar or polymer residues. The reducing power of the reagents was determined from the samples numbered 2, and the reducing power of the reaction mixture was obtained from the number 3 samples. The effect of the enzyme on the sugar or polymer was evaluated by subtracting the reducing power of Samples 1 and 2 from Sample 3. It was found that galactose and galactoglucomannan were oxidized by galactose oxidase, but glucose and mannose were not.

TABLE XIV

REAGENTS IN ENZYME OXIDATION

Samples numbered 1:

2 ml. distilled water

1 ml. sugar or polymer solution, 1-2.5 mg./ml.

Samples numbered 2:

0.06 ml. catalase

1 ml. distilled water

1 ml. phosphate buffer, pH 7

1 ml. enzyme solution, 2.7 units/ml.

Samples numbered 3:

0.06 ml. catalase

1 ml. phosphate buffer, pH 7

1 ml. sugar or polymer solution, 1-2.5 mg./ml.

1 ml. enzyme solution, 2.7 units/ml.

APPENDIX III

PAPER CHROMATOGRAPHY

SOLVENTS

Solvent A: 9:2:2 (V:V) ethyl acetate:acetic acid:water (45)

Solvent B: 8:2:1 (V:V) ethyl acetate:pyridine:water (46)

Solvent C: 18:3:1:4 (V:V) ethyl acetate:acetic acid:formic acid:water (45)

DETECTION REAGENTS

Dip: The chromatograms were consecutively dipped in solutions 1, 2, and 3 with air drying between solutions. A final dip in distilled water is recommended but not necessary.

Solutions: (1) 30 g. silver nitrate, 50 ml. distilled water, and 950 ml. acetone; (2) 20 g. sodium hydroxide, 50 ml. distilled water, and 950 ml. absolute alcohol; (3) 100 g. sodium thiosulfate and 900 ml. distilled water (47).

Spray: The chromatograms were sprayed, air dried, and heated at approximately 105°C. for five minutes.

Spray: 5 g. p-anisidine hydrochloride, 50 ml. distilled water, 100 ml. absolute alcohol, and 850 ml. of n-butanol (48).